Aquatic Toxicology 195 (2018) 67–76

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Microplastics have a more profound impact than elevated temperatures on the predatory performance, digestion and energy metabolism of an Amazonian cichlid

Bin Wen[a,b,c,1], Nan Zhang[a,b,c,1], Shi-Rong Jin[a,b,c], Zai-Zhong Chen[a,b,c,*], Jian-Zhong Gao[a,b,c,*], Ying Liu[a,b,c], Han-Peng Liu[a,b,c], Zhe Xu[a,b,c]

[a] Key Laboratory of Freshwater Aquatic Genetic Resources, Ministry of Agriculture, Shanghai Ocean University, Shanghai 201306, China
[b] Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, Shanghai Ocean University, Shanghai 201306, China
[c] Shanghai Collaborative Innovation for Aquatic Animal Genetics and Breeding, Shanghai Ocean University, Shanghai 201306, China

ARTICLE INFO

Keywords:
Microplastic
Elevated temperature
Tropical fish
Predatory performance
Digestion
Energy production

ABSTRACT

Knowledge on the impacts of microplastics (MPs) pollution on freshwater environments and biota remains limited. Meanwhile, freshwater ecosystems have been threatened by elevated temperatures caused by climate change. To date, no information exists on how MPs—especially under elevated temperature conditions—affect predatory performance, digestive processes and metabolic pathways in freshwater organisms. Here, we examined MPs, elevated temperature and their combined effects on juveniles (0+ group) of an Amazonian cichlid, the discus fish (*Symphysodon aequifasciatus*). For 30 days, fish were exposed to ambient or elevated temperatures (i.e., 28 or 31 °C) in the absence or presence of MPs (i.e., 0 or 200 μg/L). The following metrics were quantified: MPs accumulation; predatory performance; and biomarkers involved in neurotransmission, digestion and energy production. The results showed that survival rate and body length were not affected by MPs, elevated temperatures or their combination. Elevated temperatures resulted in an increase in MP concentrations in fish bodies. Exposure to MPs decreased the post-exposure predatory performance (PEPP) at ambient temperatures but not at elevated temperatures. Elevated temperatures, however, had no effect on the PEPP but antagonistically interacted with MPs, leading to similar predatory performances under present and future conditions. Acetylcholinesterase (AChE) activity was only affected by MPs and decreased in the presence of MPs, indicating adverse effects in nervous and neuromuscular function and, thus, potentially in predatory performance. Trypsin activity was only influenced by MPs and decreased during exposure to MPs. Elevated temperatures or MPs alone increased the amylase activity but interacted antagonistically. Lipase activity was not influenced by either of the two stressors. In contrast, alkaline phosphatase (ALP) activity was affected by MPs or elevated temperatures alone and decreased with both stressors. Such results indicate deficits in the digestive capabilities of early-stage *S. aequifasciatus* under elevated temperature conditions and especially during exposure to MPs. Electron transport system (ETS) activity was not influenced by either of the two stressors. Both elevated temperatures and MPs alone increased LDH activity; however, the interaction between the two stressors cancelled activity but was still higher than activity in present conditions. Citrate synthase (CS) activity decreased with elevated temperature but increased during exposure to MPs. Cytochrome c oxidase (COX) activity was only influenced by MPs and increased in the presence of MPs. Thus, *S. aequifasciatus* juveniles exposed to elevated temperatures and MPs not only relied on anaerobic glycolysis for energy production but also depended on aerobic metabolism in the presence of MPs. Overall, these findings suggested that MPs showed a greater impact than elevated temperatures on the predatory performance, digestion and energy production of *S. aequifasciatus*. Nevertheless, juvenile survival and growth were minimally impacted, and thus, *S. aequifasciatus* could cope with near-future temperature increases and MP exposure.

* Corresponding authors at: Key Laboratory of Freshwater Aquatic Genetic Resources, Ministry of Agriculture, Shanghai Ocean University, Shanghai 201306, China.
E-mail addresses: chenzz@shou.edu.cn (Z.-Z. Chen), jzhgao@shou.edu.cn (J.-Z. Gao).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.aquatox.2017.12.010
Received 27 October 2017; Received in revised form 22 December 2017; Accepted 24 December 2017
Available online 26 December 2017
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1. Introduction

When present in the environment, microplastics (MPs, particles < 5 mm, Thompson et al., 2009) become a threat to biodiversity because they can cause toxic and physical effects on biota. Even though numerous studies are available on the effects of MPs in marine ecosystems, our understanding of the impacts of MPs on freshwater environments is still limited. In recent years, research efforts have focused on trying to describe MP pollution and its impacts on freshwater ecosystems worldwide (e.g., Wagner et al., 2014; Eerkes-Medrano et al., 2015; Silva-Cavalcanti et al., 2017). Nevertheless, major knowledge gaps remain on the interactions between MPs and freshwater biota and the biological consequences of ingesting MPs (Eerkes-Medrano et al., 2015; Silva-Cavalcanti et al., 2017).

At the same time, anthropogenic environmental changes will likely cause increased episodes of high temperatures in many freshwater habitats, with forecasts estimating an increase in the range of 1.4–3.1 °C by the year 2100 (IPCC, 2014). Such increases in water temperature can directly influence a range of physiological processes and, ultimately, the persistence and survival of extant species (e.g., Ganser et al., 2015; Jesus et al., 2017). Therefore, freshwater organisms and biodiversity are facing threats posed by the simultaneous presence of MPs and elevated temperature events. It is necessary to consider combined effects when evaluating the impacts of environmental changes on organisms; this is because the interactive effects are not only the simple sum of the two independent responses but may also be synergistic or antagonistic (Crain et al., 2008). Subsequently, to understand whole-organism and eventual ecosystem-level consequences, it becomes important to investigate the physiological and biochemical pathways of freshwater organisms that are threatened by the combined effects of MPs and elevated temperature. This is especially true for species that are likely to experience these impacts more rapidly, such as those inhabiting the Amazon region.

The Amazon is an extremely large and diverse environment, which is characterized by variable water temperature, low levels of dissolved oxygen, and acidic pH associated with reduced ion concentrations (Val and De Boeck, 2017); additionally, the Amazon supports massive biological diversity with more than 3000 known fish species (Junk et al., 2007; Alber et al., 2011; Castello et al., 2013). These species have developed a wide range of adaptations across different levels of biological organization, enabling them to thrive in these dynamic and extreme environments (e.g., De Almeida-Val et al., 2005; Duarte et al., 2013; Val et al., 1998, 2015; Maccormack et al., 2017). Despite this, the Amazon fish fauna is threatened by a series of anthropogenic environmental disturbances, including water pollution from various sources of contaminants (e.g., Lemgruber et al., 2013; Kochhann et al., 2013; Braz-Mota et al., 2017; Lucena et al., 2017; Souza-Bastos et al., 2017), and threats resulting from climate change (e.g., Prado-Lima and Val, 2016; de Oliveira and Val, 2017; Campos et al., 2017). These environmental stressors may force organisms outside their tolerance boundaries, which compromises the overall fitness and survival of local populations. Additionally, the Amazon was reported to be among the top twenty most-polluted rivers, as predicted by the global river plastic inputs model (Lebreton et al., 2017). However, studies on the effects of MPs on fish species, especially under a warming environment, have been scarce to date.

Organisms generally reduce their available energy in stressful environmental conditions, and as a result, their feeding, digestion and energy metabolism may experience functional disturbances (Hochachka and Somero, 2002). When exposed to elevated temperatures, food consumption and foraging activities by fish may increase or remain unchanged (Ferrari et al., 2015; Nagelkerken and Munday, 2016). In contrast, fish that ingest MPs may reduce their activity and predatory performance (Mattsson et al., 2015; de Sá et al., 2015; Lönnstedt and Eklöv, 2016), which is likely to result in a lower food intake by the fish and a reduction in their individual fitness, which has potential population-level effects. The activities of digestive enzymes are expected to be affected by external factors that modify metabolic functions, such as temperature and pH (Stumpp et al., 2013; Pimentel et al., 2015). Digestive enzyme activities in fish have been reported to decrease as a result of warmer temperatures (Rosa et al., 2014, 2016). Additionally, ingested MPs can cause injuries to the digestive tract and create a sense of false food satiation (Von Moos et al., 2012; Pedà et al., 2016) and result in increased or unchanged digestive enzymes activities (Karami et al., 2016; Haghi and Banaee, 2017; Romano et al., 2017), which in turn, may affect the ability of fishes to digest and absorb nutrients. Moreover, the expected elevated temperatures have the potential to decrease or increase energy production, though this process is dependent on the fish species and their environmental conditions (Prado-Lima and Val, 2016; Jesus et al., 2017); while the ingestion of MPs by fish has also been shown to induce changes in energy metabolism (Cedervall et al., 2012; Mattsson et al., 2015), which may negatively affect the subsequent cellular processes necessary for fish survival. The early juveniles of fish have crucial direct roles in the development of their species' populations, and juveniles also have indirect roles in the entire ecosystem since they provide top-down control of prey populations (Oliveira et al., 2013). However, until now, how the feeding performance, digestive capabilities and energy metabolism change in fish in early life stages due to the combined effects of future elevated temperature and MP exposure is unknown.

Here, we intend to discern the physiological responses of Amazon fish, using a discus fish (*Symphysodon aequifasciatus*), to projected future climate change scenarios predicting elevated temperatures (+3 °C), exposure to MPs (200 μg/L) and their combination. *S. aequifasciatus* is a native cichlid species of the Amazon basin and is amongst the most common fish in warm water (Junk et al., 2007). Given the narrow thermal window (24–33 °C) in which *S. aequifasciatus* exists, we hypothesized that this stenotherm might display a significant decrease in physiological performance when confronted with an increase in temperature and exposure to MPs. To test this hypothesis, we used early life-stage *S. aequifasciatus* individuals to investigate a comprehensive set of physiological and biochemical responses, which included i) accumulation of MPs in fish bodies; ii) post-exposure predatory performance (PEPP); iii) acetylcholinesterase (AChE) activity); iv) digestive enzymes activities (i.e., trypsin, lipase, amylase and alkaline phosphatase (ALP)); and v) energy metabolism related enzymes activities (i.e. electron transport system (ETS), lactate dehydrogenase (LDH), citrate synthase (CS) and cytochrome c oxidase (COX)). PEPP was selected as an effect criterion because predation is a crucial function for the performance and survival of fish in the wild (de Sá et al., 2015; Ferreira et al., 2016). AChE, which is responsible for the degradation of the neurotransmitter acetylcholine in cholinergic synapses, was used as an indicator of neurotoxicity (Ferreira et al., 2016). Trypsin, lipase, amylase and ALP activities were used as indicators of changes in digestive capabilities (Pimentel et al., 2015; Rosa et al., 2016). ETS, LDH, CS and COX activities were selected as indicators of modifications of metabolic pathways under sub-optimal conditions (O'Brien and Mueller, 2010; Campos et al., 2017). Assessing these endpoints may provide insight into the underlying processes that may indicate deteriorations in the conditions of the organism before these effects are observed at the population level.

2. Materials and methods

2.1. Characterization of MPs

Fluorescent polyethylene plastic microspheres (particle sizes 70–88 μm), hereafter termed MPs, were purchased from Tianjin BaseLine ChromTech Research Centre (Tianjin, China) in a distilled water carrier. According to the manufacturer's information, the MP basic properties are: diameter of 70–88 μm; density of 10 mg/mL; excitation wavelength of 488 nm; and emission wavelength of 518 nm.

The MPs were stained by gradual solvent evaporation method using NBD-Cl as the fluorescent dye (Zhang et al., 2009). The suspensions of MPs were prepared using UV-sterilized aerated water and were sonicated prior to use. The aggregation of MPs in the water was determined by dynamic light scattering (DLS) (Malvern, ZEN1600, UK), and no significant aggregation was observed.

2.2. Fish breeding

Adult discus fish, *S. aequifasciatus*, were collected from a population located on Lake Manacapuru (Manacapuru River) in the central Amazon basin (03°17′S; 60°37′W). The obtained fish were held at the Ornamental Fish Breeding Laboratory of Shanghai Ocean University (Shanghai, China). Experimental fish were cultured in aerated tanks (100 × 60 × 50 cm, 300 L) equipped with a recirculation system. Water temperature was maintained at 28.0 ± 1.0 °C, pH remained between 5.5–6.5, dissolved oxygen was > 6.0 mg L^{-1}, and a 12:12 h (L/D) photoperiod was used. The fish were fed *ad libitum* with a diet consisting of beef heart, duck heart and shrimp meat, which was named 'beef heart hamburger'. Breeding pairs of *S. aequifasciatus* were taken from the same stock tank and introduced into a 'spawning tank' (35 × 60 × 50 cm, 105 L), which contained a block (5 × 10 × 10 cm) as a spawning site. When parental fish spawned (100–300 eggs per clutch), they were regarded as a formed breeding pair. When spawning and breeding started, the contrast between the white line and the body coloration became more pronounced than before spawning and breeding activities started. Newly hatched *S. aequifasciatus* fed on parental epidermal mucus, which is essential for the growth and development of free-swimming larvae (Maunder et al., 2013).

2.3. Experimental design

At one week post-hatch, which was one week before the beginning of the experiment, a total of 240 full-sibling juvenile *S. aequifasciatus* individuals were randomly distributed among 50-L tanks (20 fish per tank, three tanks per treatment) and fed three times a day with newborn (24-h old) *Artemia* nauplii. Exposure was executed according to the guidelines (OECD, 1992) with minor modifications. Pirhonen et al. (2014) suggested that juvenile *S. aequifasciatus* showed optimal growth performance at temperatures of 28–29 °C. An elevated temperature of 31 °C, an increase of 3 °C, reflects the projected near-future temperature increase (IPCC, 2014). Hence, in this study, juvenile *S. aequifasciatus* fish were acclimated to a temperature of 28 or 31 °C to investigate long-term acclimation to temperature outside the preferred range of this species. MPs with particle sizes < 100 μm were generally employed in toxicological studies (e.g., Karami et al., 2016; Lönnstedt and Eklöv, 2016). No information is available on the concentrations of MPs smaller than 100 μm in Amazon ecosystems. The MP concentrations (200 μg/L) used in the current study were within the range employed by earlier toxicity testing (e.g., Oliveira et al., 2013; Karami et al., 2016, 2017). The experiment was composed of one treatment combination with the following 2 × 2 design: fish were exposed to the control or elevated temperature (i.e., 28 vs. 31 °C) crossed with the absence or presence of MPs (i.e., 0 vs. 200 μg/L).

Water quality parameters (e.g., ammonia < 0.02 mg L^{-1}, nitrite < 0.01 mg L^{-1} and pH 5.5–6.5) were monitored using an YSI ProPlus Multi-parameter WQ Meter, USA. All tanks were continuously aerated to ensure comparable O_2 saturation levels and to minimize the aggregation of MPs. Test media were changed every day with UV-treated water spiked with appropriate MP concentrations. The juveniles were maintained at a 12:12 h L/D photoperiod throughout the exposure period. After 30 days of experimental exposure, fish survival and individual body lengths were determined, after which fish were sacrificed by anaesthesia with tricaine methanesulfonate (MS-222) followed by decapitation, in accordance with the American Veterinary Medical Association guidelines for animals (Leary et al., 2013). Early *S. aequifasciatus* juveniles are very sensitive to manipulation. Thus, the total length of the fish used in the experiments was only determined at the end of the exposure period (i.e., after assessing their PEPP). Immediately after the sacrifice of each fish, the head and body tissues were isolated on ice, frozen in liquid nitrogen, and stored at −80 °C for further analyses. All fish used in this study were housed and sacrificed according to the approved animal use protocols dictated by the Institutional Animal Care and Use Committee at the Shanghai Ocean University (SHOU IACUC protocol # 20171015).

2.4. Determining concentrations of MPs in fish bodies

The accumulation of MPs in whole-fish bodies was determined using the method described in Lu et al. (2016). After lyophilization for 72 h, the whole-fish bodies were digested in nitric acid (1 mL) at 70 °C for 2 h and then diluted with deionized water to a final volume of 5 mL. The concentration of MPs in fish tissue was measured using a Synergy™ Neo2 Multi-Mode Microplate Reader with excitation at 488 nm and emission at 518 nm. The standard curve was generated by using serial dilutions of fluorescent MP suspensions. Fluorescently labelled microspheres were used in this study to quantify the accumulation of MPs in fish bodies (Watts et al., 2014; Lu et al., 2016). As such, the accumulation of MPs in the whole-bodies of fish not exposed to MPs was not reported. However, to eliminate any positive error produced by fish, the background fluorescence of the bodies of fish not exposed to MPs was measured in the same way as for fish exposed to MPs, and these values were subtracted from the values of the treatment samples.

2.5. Post-exposure predatory performance assay

The PEPP assay was assessed individually at the end of the exposure period, as indicated in Luís et al. (2015). The new-born (24-h old) nauplii were offered as prey for fish. Each fish was transferred to a prey-exposure 300-mL chamber (diameter of 20 cm). To exclusively examine the post-exposure effect, the fish were not fasted prior to the assay. After a 5-min acclimation period, 15 live nauplii (A) were added to the chamber using a 1-mL Pasteur pipet. After another 5-min feeding period, each fish was carefully removed and placed in another similar chamber without adding nauplii; this enabled the determination of whether uneaten nauplii were accidentally removed with the fish. The number of nauplii remaining in the prey-exposure chamber was counted (B) by visual observation to confirm the number of prey ingested. The number of nauplii captured and ingested by each fish (C) was calculated as: C = A − B. The predatory performance was expressed as the percentage of prey ingested by the fish relative to the initial number of prey offered (C × 100/A).

2.6. Determination of acetylcholinesterase level

Samples of head tissue were homogenized (1:9, w/v) in a potassium-phosphate buffer (0.1 M, pH 7.5). The homogenate solution was centrifuged at 3000*g* at 4 °C for 10 min, and the supernatants were collected for measuring AChE activity using detection kits (Nanjing Jiancheng, Bioengineering Institute, Nanjing, China). AChE activity was determined according to Ellman et al. (1961) using acetylthiocholine as substrate. The rate of acetylthiocholine degradation was assessed at 412 nm by measuring the increase in the yellow colour caused by the binding of thiocholine with 5,5-dithio-bis-(2-nitrobenzoic acid). One unit of AChE activity corresponded to the hydrolysis of 1 μmol of substrate per 6 min per mg of protein tissue at 37 °C. The specific AChE activity was expressed as unit per mg protein. Protein content was quantified according to the Bradford (1976) method, and bovine serum albumin was used as the standard.

2.7. Measurement of digestive enzyme activity

Samples of body tissue were homogenized (1:9, w/v) in a potassium-phosphate buffer (0.1 M, pH 7.5). The homogenate solution was further centrifuged at 3000*g* at 4 °C for 10 min, and the supernatants were collected for measuring the activities of digestive enzymes using corresponding detection kits (Nanjing Jiancheng, Bioengineering Institute, Nanjing, China).

Trypsin activity was determined according to Lowry et al. (1951) using Folin phenol reagent. One unit of trypsin activity was defined as the amount of enzyme that caused an increase in absorbance of 0.003 at 253 nm per min per mg of protein tissue at 37 °C. The specific trypsin activity was expressed as unit per mg protein.

Lipase activity was determined according to the method described in Shihabi and Bishop (1971), and triglycerides were used as substrate. One unit of lipase activity corresponded to the hydrolysis of 1 μmol of substrate per min per mg of protein tissue at 37 °C. The specific lipase activity was expressed as unit per mg protein.

Amylase activity was quantified according to Metais and Bieth (1968), and soluble starch-iodine (0.1 M) was used as substrate. One unit of amylase activity was defined as the amount of enzyme required for hydrolysing 10 mg of starch at 37 °C within 30 min. The specific amylase activity was expressed as unit per mg protein.

ALP activity was quantified according to Hausamen et al. (1967), and disodium phenyl phosphate was used as substrate. One unit of amylase activity was defined as the amount of enzyme required for producing 1 mg of phenol at 37 °C within 15 min. The specific amylase activity was expressed as unit per mg protein.

2.8. Measurement of metabolic enzyme activity

Samples of body tissue were homogenized (1:9, w/v) in a potassium-phosphate buffer (0.1 M, pH 7.5). The homogenate solution was further centrifuged at 3000*g* at 4 °C for 10 min, and the supernatants were collected for measuring the activities of energy metabolism-related enzymes using corresponding detection kits (Nanjing Jiancheng, Bioengineering Institute, Nanjing, China).

ETS activity was measured according to the method described by De Coen and Janssen (1997). Briefly, the samples were homogenized in homogenising buffer (0.1 M Tris–HCl, 0.2% (w/v) Triton X-100, pH 8.5, 75 μM $MgSO_4$ and 0.15% (w/v) polyvinylpyrrolidone). After centrifugation for 10 min (4 °C, 3000*g*), 100 μL of supernatant from each extract was added into 300 μL of substrate solution (0.1 M Tris–HCl, 0.2% Triton X-100, pH 8.5 and 1.7 mM NADH, 0.25 mM NADPH). The reaction was started by adding 100 μL of 8 mM *p*-iodonitrotetrazolium and proceeded at 20 °C for 10 min; the reaction was then stopped by adding 100 μL of quench solution (50% formalin and 1 M H_3PO_4). The resulting increase in absorbance was measured at 490 nm. The formation of 2 mmol formazan equals the consumption of 1 mmol O_2 in the ETS. The resulting energy consumption was then converted into caloric values using the oxyenthalpic equivalents of 484 kJ/mol O_2 for the average mixture of protein, lipid and carbohydrate (Gnaiger, 1983).

LDH activity was assayed using 0.1 M phosphate buffer (pH 7.5) and 0.2 mM NADH solution in 0.1 M phosphate buffer. The reaction was initiated by adding 0.2 mM sodium pyruvate, and the absorbance was recorded at 340 nm (Wrobleuiski and Ladue 1955). One unit of LDH activity was defined as the amount that produced 1 μmol of pyruvate after 15 min at 37 °C. The specific LDH activity was expressed as unit per mg protein.

CS activity was detected according to Sidell et al. (1987) in a buffer containing 75 mmol L^{-1} Tris–HCl, 0.25 mmol L^{-1} DTNB, 0.4 mmol L^{-1} acetyl-CoA and 0.5 mmol L^{-1} oxaloacetate. The activity was determined from the increase in absorbance at 412 nm, which was caused by the transfer of sulfhydryl groups from coenzyme A to 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). The specific CS activity was expressed as unit per mg protein.

COX activity was determined according to a protocol modified from Moyes et al. (1997) in buffer containing 20 mmol L^{-1} Tris–HCl, 0.05% Tween 20 and 0.057 mM reduced cytochrome c at pH = 8.0. The activity was determined from the decrease in extinction at 450 nm through oxidation of cytochrome c. The specific COX activity was expressed as unit per mg protein.

2.9. Calculation and statistical analysis

Raw data were assessed for the normality of distribution and the homogeneity of variance with the Kolmogorov-Smirnov test and Levene's test, respectively. The data conformed to a normal distribution and were suitable for testing with analysis of variance (ANOVA). The differences in the accumulation of MPs in fish between treatments were compared with Student's *t*-test at the significance level of 0.05 ($p < 0.05$). The interactive effects of elevated temperature and MPs on all other measured parameters were assessed by two-way ANOVA. Factors detected to be significant were further analysed using Bonferroni's multiple comparison tests. Data analyses were performed using the software SPSS for Windows (Release 20.0).

3. Results

3.1. Growth performance

Both elevated temperature and exposure to MPs showed no significant effects on survival rate (SR) and body length (BL) (Table 1). No significant differences in SR and BL were observed between treatments (Fig. 1A and B).

Table 1
Two-way ANOVA results on effects of temperature (T) and microplastics (MPs) on all measured parameters of discus fish *Symphysodon aequifasciatus*. SR = survival rate, BL = body length, AChE = acetylcholinesterase, PEPP = post-exposure predatory performance, ALP = alkaline phosphatase, ETS = electron transport system, LDH = lactate dehydrogenase, CS = citrate synthase and COX = cytochrome c oxidase.

Source		Measured parameters											
		SR	BL	AChE	PEPP	Trypsin	Lipase	Amylase	ALP	ETS	LDH	CS	COX
T	*df*	1	1	1	1	1	1	1	1	1	1	1	1
	F	0.036	0.385	2.014	1.389	0.007	2.891	24.180	924.654	0.151	1.883	13.937	2.229
	p	0.855	0.539	0.194	0.272	0.938	0.127	**0.001**	**< 0.001**	0.708	0.207	**0.006**	0.174
MPs	*df*	1	1	1	1	1	1	1	1	1	1	1	1
	F	0.036	0.070	73.159	9.389	113.070	1.629	10.192	91.655	1.451	2.131	7.448	7.688
	p	0.855	0.792	**< 0.001**	**0.015**	**< 0.001**	0.238	**0.013**	**< 0.001**	0.263	0.183	**0.026**	**0.024**
T × MPs	*df*	1	1	1	1	1	1	1	1	1	1	1	1
	F	0.893	1.155	0.005	9.389	2.846	0.604	64.231	3.248	2.893	8.612	2.911	0.755
	p	0.372	0.291	0.948	**0.015**	0.130	0.459	**< 0.001**	0.109	0.127	**0.019**	0.126	0.410

Bold indicates $p < 0.05$.

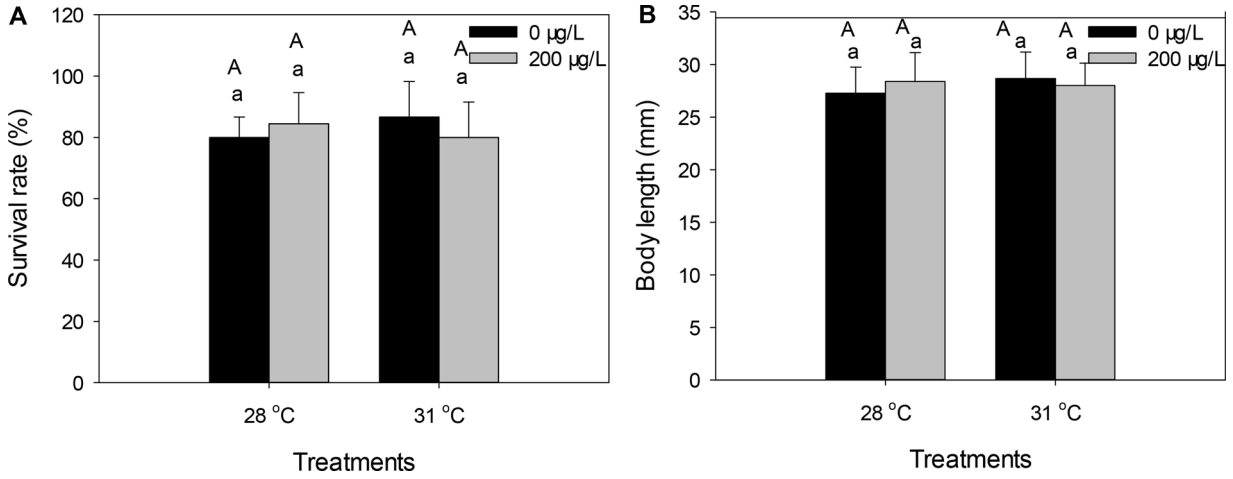

Fig. 1. Growth of juvenile *Symphysodon aequifasciatus* exposed to ambient (28 °C) or elevated temperatures (31 °C) crossed with the absence (0 μg/L, dark bars) or presence of MPs (200 μg/L, light bars). The same lowercase letters indicate no differences between concentrations of MPs within each temperature group ($p > 0.05$). The same uppercase letters indicate no significant differences between temperatures within each concentration of MPs ($p > 0.05$).

3.2. Accumulation of MPs in fish bodies

After 30 days of experimental exposure, elevated temperature significantly increased the concentrations of MPs in the whole body of fish exposed to MPs (Fig. 2).

3.3. Post-exposure predatory performance

The PEPP was significantly influenced by MPs alone and by the interaction between elevated temperature and MPs (Table 1). Exposure to MPs significantly decreased the PEPP at ambient temperatures, but not at elevated temperatures. Elevated temperatures showed no significant effect on PEPP in the absence or the presence of MPs (Fig. 3).

3.4. Acetylcholinesterase activity

Head AChE activity was significantly affected by exposure to MPs alone and showed a decrease in the presence of MPs (Table 1; Fig. 4).

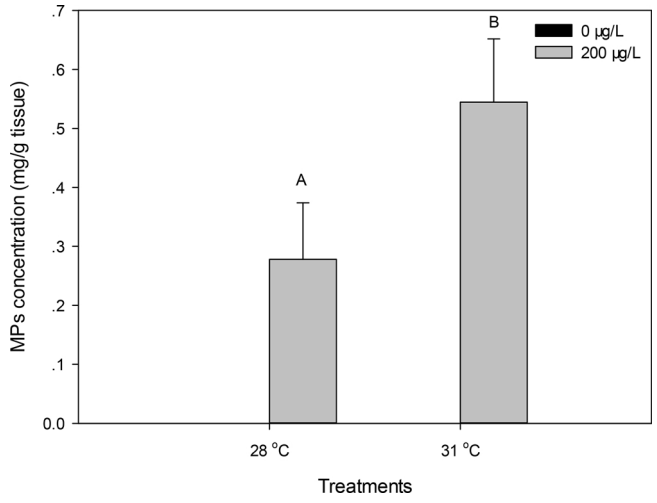

Fig. 2. Concentration of MPs in the whole body of *S. aequifasciatus* juveniles exposed to ambient (28 °C) or elevated temperatures (31 °C) crossed with the absence (0 μg/L, not shown) or presence of MPs (200 μg/L, light bars). Different uppercase letters indicate significant differences between ambient and elevated temperatures in the presence of MPs ($p < 0.05$).

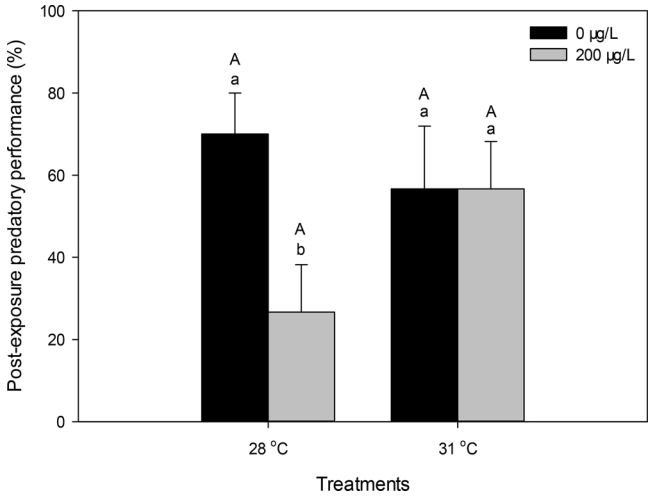

Fig. 3. Post-exposure predatory performance (PEPP) of *S. aequifasciatus* juveniles exposed to ambient (28 °C) or elevated temperatures (31 °C) crossed with the absence (0 μg/L, dark bars) or presence of MPs (200 μg/L, light bars). Different lowercase letters indicate significant differences between MP concentrations within each temperature ($p < 0.05$). Different uppercase letters indicate significant differences between temperatures within each MP concentration ($p < 0.05$).

3.5. Digestive enzymes activities

Trypsin activity was significantly affected only by MPs and appeared to significantly decrease in the presence of MPs (Table 1; Fig. 5A). Neither elevated temperatures nor MPs had significant effects on lipase activity (Table 1), and there were no significant differences between treatments (Fig. 5B). Amylase activity was significantly influenced by elevated temperatures, MPs and the interaction of elevated temperatures and MPs (Table 1). Exposure to MPs significantly increased amylase activity at ambient temperatures but significantly decreased amylase activity at elevated temperatures. Elevated temperatures significantly increased amylase activity in the absence of MPs but significantly decreased amylase activity in the presence of MPs (Fig. 5C). ALP activity was significantly affected by elevated temperature alone and MPs alone (Table 1). Independently, both elevated temperature and MPs significantly decreased ALP activity (Fig. 5D).

3.6. Metabolic enzymes activities

Neither elevated temperatures nor exposure to MPs had significant

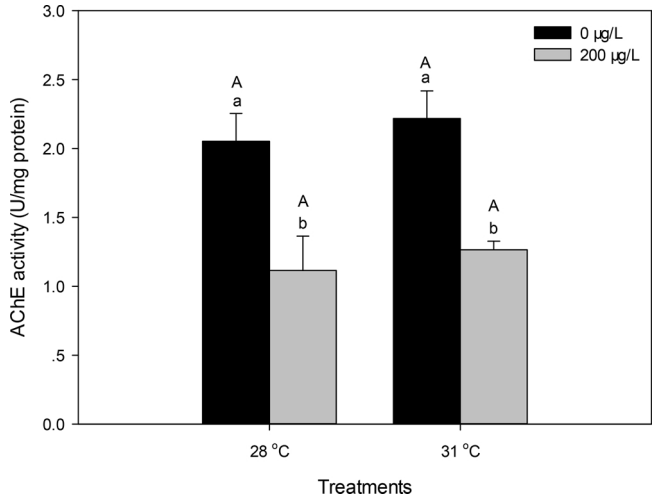

Fig. 4. Acetylcholinesterase (AChE) activity of *S. aequifasciatus* juveniles exposed to ambient (28 °C) or elevated temperatures (31 °C) with the absence (0 µg/L, dark bars) or presence of MPs (200 µg/L, light bars). Different lowercase letters indicate significant differences between MP concentrations within each temperature ($p < 0.05$). Different uppercase letters indicate significant differences between temperatures within each MP concentration ($p < 0.05$).

effects on ETS activity (Table 1), and there were no significant differences between treatments (Fig. 6A). LDH activity was significantly influenced by the interaction of elevated temperature and MPs (Table 1). Exposure to MPs significantly increased LDH activity at ambient temperatures but significantly decreased LDH activity at elevated temperatures. Elevated temperatures significantly increased LDH activity in the absence of MPs but significantly decreased LDH activity in the presence of MPs (Fig. 6B). CS activity was significantly affected by elevated temperatures alone and MPs alone (Table 1). Elevated temperatures significantly decreased CS activity, but MPs significantly increased CS activity (Fig. 6C). COX activity was significantly influenced by MPs alone (Table 1) and showed a significant increase in the presence of MPs (Fig. 6D).

4. Discussion

Temperature can play a significant role in fish condition (Strobel et al., 2012; Enzor et al., 2017). Enzor et al. (2017), for example, reported decreased growth rates in notothenioid fish acclimated to warm water despite being fed to satiation. Among other potential effects, the ingestion of MPs instead of food may also lead to delayed growth delay and increased mortality (e.g., due to starvation), with negative effects on population fitness (Luís et al., 2015). Environmental changes are likely to endanger regions with high biodiversity, especially areas like the Amazon region, which is home to a significant portion of the world's biodiversity. However, no significant effects on the survival and growth of *S. aequifasciatus* juveniles were experienced by elevated temperatures, exposure to MPs, or the combination of both factors. Similarly, the growth of zebrafish (*Danio rerio*) larvae was not

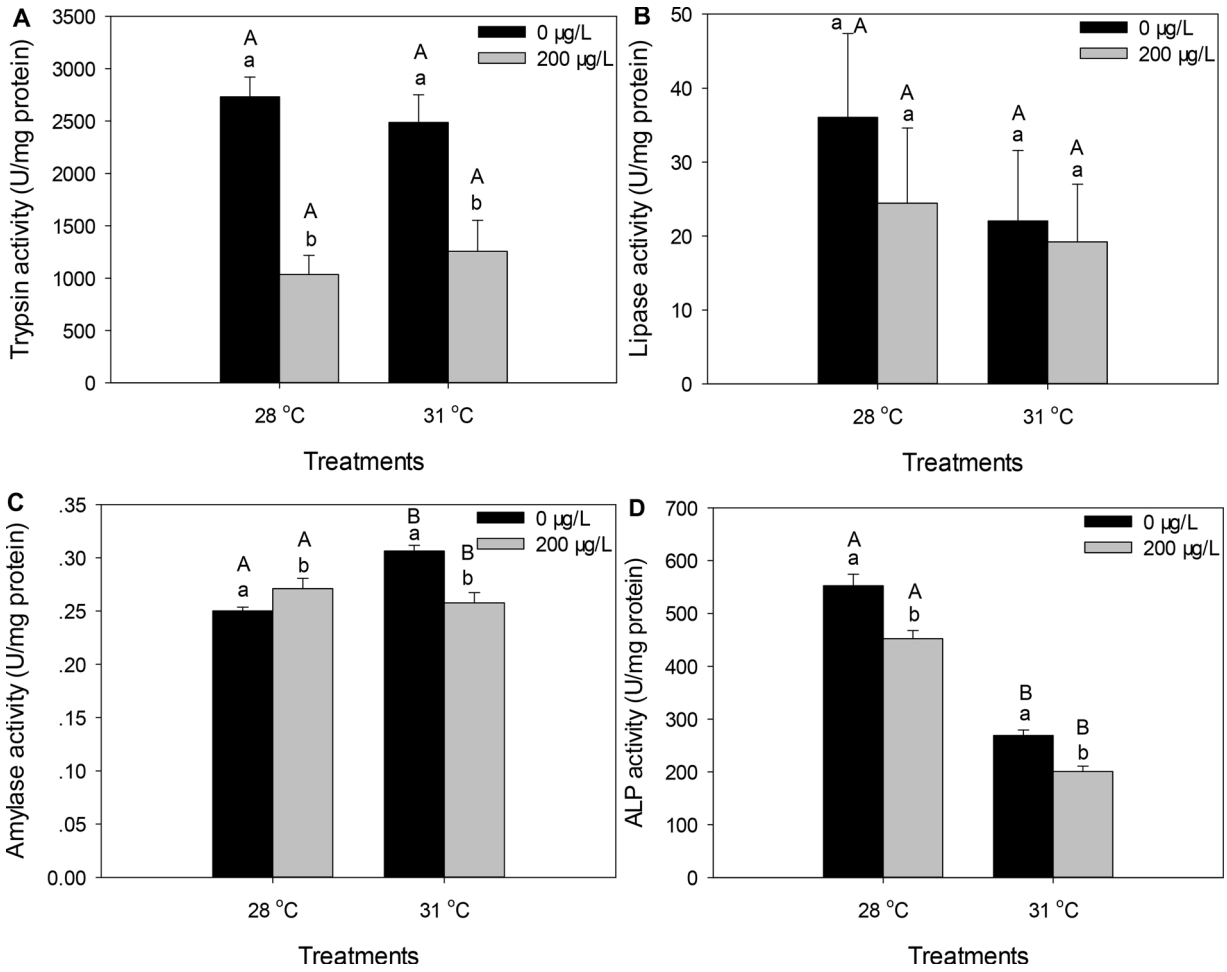

Fig. 5. Digestive enzymes activities of *S. aequifasciatus* juveniles exposed to ambient (28 °C) or elevated temperatures (31 °C) crossed with the absence (0 µg/L, dark bars) or presence of MPs (200 µg/L, light bars). (A) trypsin, (B) lipase, (C) amylase and (D) alkaline phosphatase (ALP). Different lowercase letters indicate significant differences between MP concentrations within each temperature ($p < 0.05$). Different uppercase letters indicate significant differences between temperatures within each MP concentration ($p < 0.05$).

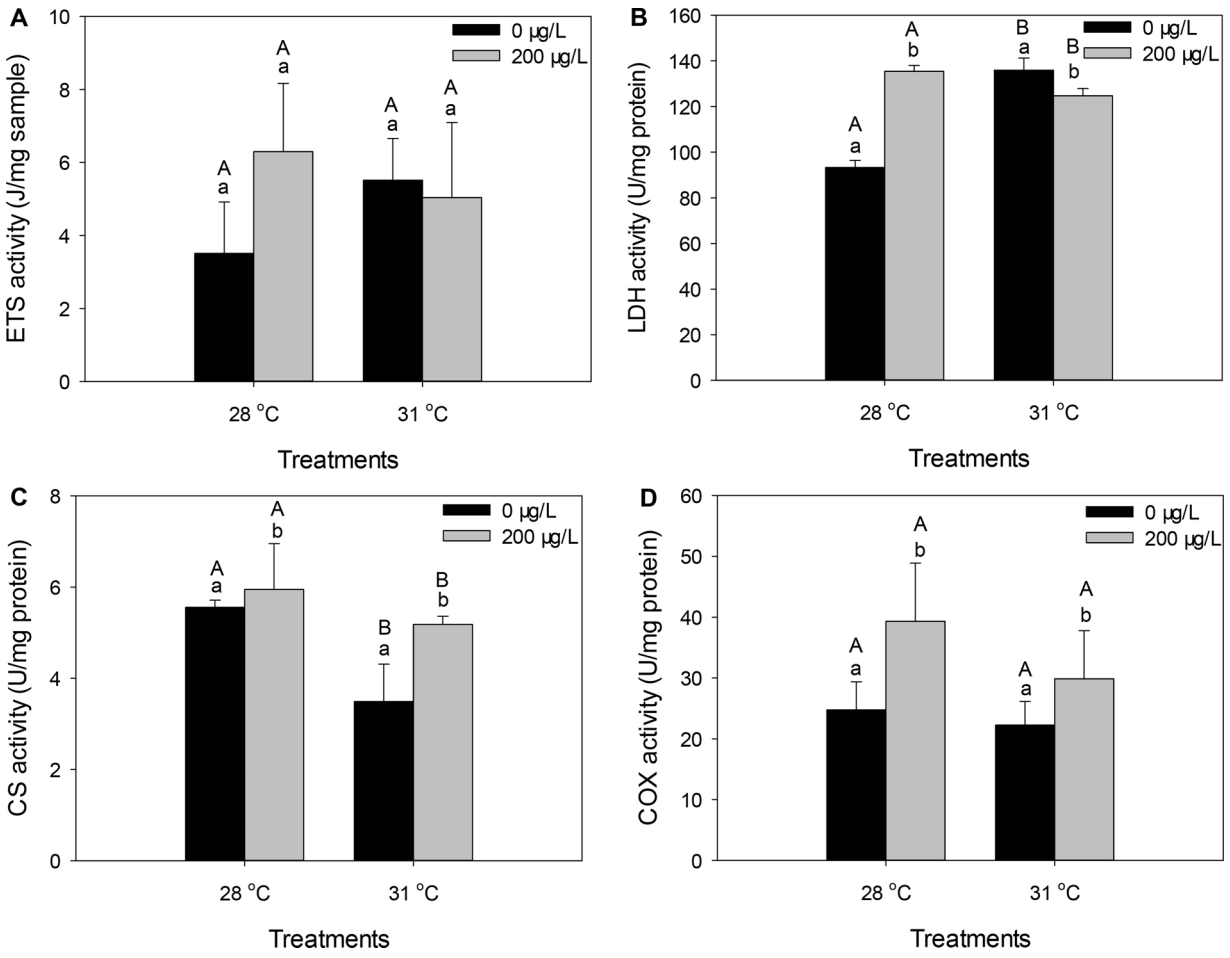

Fig. 6. Metabolic enzymes activities of *S. aequifasciatus* juveniles exposed to ambient (28 °C) or elevated temperature (31 °C), crossed with the absence (0 μg/L, dark bars) or presence of MPs (200 μg/L, light bars). (A) electron transport system (ETS), (B) lactate dehydrogenase (LDH), (C) citrate synthase (CS) and cytochrome c oxidase (COX). Different lowercase letters indicate significant differences between MP concentrations within each temperature ($p < 0.05$). Different uppercase letters indicate significant differences between temperatures within each MP concentration ($p < 0.05$).

influenced by MPs (Karami et al., 2017). Such results suggest that *S. aequifasciatus* may be capable of ingesting sufficient food to meet its required energy demand and do not experience a decrease in scope for growth; thus, this species can survive in the projected near-future temperatures (+3 °C) and in water with certain concentrations of MPs (200 μg/L).

Though the survival and growth of juvenile *S. aequifasciatus* was minimally impacted, the biological accumulation of MPs in fish bodies exposed to MPs was profound. Similarly, the uptake and retention of MPs has also been observed in other species (e.g., Watts et al., 2014; Lu et al., 2016). Moreover, elevated temperatures resulted in an increase in the accumulation of MPs in fish bodies. As such, the risks of adverse effects on *S. aequifasciatus* individuals and population fitness resulting from exposure to MPs might increase under elevated temperature conditions. This may be supported by the study of Fonte et al. (2016), who found that rising temperature increased the toxicity of MPs to common goby (*Pomatoschistus microps*) juveniles (Fonte et al., 2016). Therefore, it is necessary to consider multi-stressor effects when evaluating the impacts of environmental changes on fish.

MPs alone significantly decreased the PEPP. Similarly, de Sá et al. (2015) showed that the presence of MPs in water could reduce the predatory performance and efficiency of *P. microps* juveniles. Luís et al. (2015) found that MPs alone induced a decrease in the predatory performance of *P. microps*. Fonte et al. (2016) also reported the inhibition of PEPP in *P. microps* exposed to MPs. In the wild, a decrease in predatory performance likely results in a reduced capability of juveniles to capture prey and escape predators (Ferreira et al., 2016). Because the success of the juvenile phase is crucial for population growth, fish populations exposed to MPs may experience reductions in individual fitness, which likely results in declines in population fitness if a significant number of juveniles are affected (de Sá et al., 2015; Fonte et al., 2016). The growth and survival of *S. aequifasciatus* juveniles, however, was not influenced by exposure to MPs, indicating that the decreased PEPP might not lead to starvation and/or delayed growth in juveniles. Elevated temperatures, however, had no effect on the PEPP but antagonistically acted with MPs, leading to similar predatory performance under present and future conditions.

The PEPP was assessed through the percentage of *Artemia* nauplii ingested by fish, which is a process that involves prey perception, capture and ingestion. Thus, the process is complex and may be disrupted by toxic effects, especially effects on the nervous system that negatively affect the visual capability, movement coordination and/or neuromuscular performance of the fish (Ferreira et al., 2016). AChE can provide valuable information regarding neuronal functions crucial for the survival and performance of juveniles. In fish, inhibition of AChE activity by 30% or more may lead to disruption of the nervous system function (Vieira et al., 2009; Almeida et al., 2010), which has a wide range of adverse effects at the individual level as a result of intoxication (e.g., reduction of swimming performance or delayed growth) and can potentially lead to death. MPs individually caused significant reductions in AChE activity, indicating adverse effects in cholinergic neurotransmission and, thus, potentially in nervous and neuromuscular function. Similarly, in *P. microps* juveniles exposed to MPs alone, the inhibition of AChE activity was detected (Oliveira et al., 2013; Luís

et al., 2015; Fonte et al. (2016). Deng et al. (2017) also reported the activity of AChE in mice livers decreased after exposure to MPs. On the other hand, decreased AChE may indicate the ability of MPs to negatively interfere with behaviours that are fundamental for survival and performance, such as predatory performance, which is supported by the decrease in PEPP during exposure to MPs. Elevated temperatures, however, had no significant effects on PEPP and AChE activity. Similarly, Ferreira et al. (2016) found elevated temperatures had no effects on these parameters in *P. microps*. These results indicate that MPs could induce serious neurotoxicity and reduce predatory performance more than elevated temperature.

In addition to affecting the predatory performance of juveniles, elevated temperatures and exposure to MPs also affected the activities of digestive enzymes. Trypsin activity showed a clear decrease in the presence of MPs. In contrast, trypsin activity increased in silver barb *Barbodes gonionotus* fry exposed to MPs (Romano et al., 2017). Elevated temperatures, however, had no impact on trypsin activity. Similarly, the trypsin activity of flatfish larvae, *Solea senegalensis*, was not influenced by elevated temperatures (Pimentel et al., 2015). In contrast, juvenile bamboo shark (*Chiloscyllium punctatum*) experienced a significant increase in trypsin activity under elevated temperature conditions (Rosa et al., 2016). Exposure to MPs or elevated temperature alone increased the amylase activity, but the two variables antagonistically interacted and affected activity, leading to similar enzyme activities under present and future conditions. Both MPs and elevated temperatures alone decreased ALP activity. In contrast, common carp (*Cyprinus carpio*) exposed to MPs presented an increase in ALP activity (Haghi and Banaee, 2017), and juvenile *C. punctatum* experienced an increase in ALP activity under elevated temperature conditions (Rosa et al., 2016). The ALP activity of flatfish larvae, *S. senegalensis*, was not affected by elevated temperatures (Pimentel et al., 2015), and the ALP activity of African catfish (*Clarias gariepinus*) was not influenced by exposure to MPs (Karami et al., 2016). Overall, these results suggested some deficits in the digestive capabilities of *S. aequifasciatus* juveniles subjected to elevated temperatures, and especially when they were exposed to MPs.

The analysis of metabolic enzyme capacity after acclimation to a dual-stressor treatment provides a clearer picture whether—and to what extent—the energy metabolism and mitochondrial enzymes of *S. aequifasciatus* juveniles can compensate for elevated temperatures and exposure to MPs. LDH is an anaerobic enzyme involved in the conversion of pyruvate to lactate in glycolysis. Both elevated temperature and MPs alone increased LDH activity; however, the interaction between the two stressors cancelled activity but was still higher than in the control conditions. Hence, ATP was typically provided by anaerobic glycolysis in sustaining glycolytic flux during elevated temperature conditions, exposure to MPs, or the combination of both variables (Hochachka and Somero, 2002). Similarly, Campos et al. (2017) found that both Amazon fishes *Paracheirodon simulans* and *P. axelrodi* showed increased LDH activities in muscle tissue at elevated temperatures, indicating a direct correlation between LDH and temperature. Additionally, Deng et al. (2017) found that MPs induced significant increases in the LDH activity in the liver of mice. In contrast, Karami et al. (2016) showed that the LDH activity of African catfish (*C. gariepinus*) was not influenced by exposure to MPs. Haghi and Banaee (2017) also found common carp (*C. carpio*) exposed to MPs showed no significant changes in LDH activities. Measurements of maximal CS and COX activities are frequently used to determine changes in aerobic metabolic capacity in ectotherms in response to environmental changes (O'Brien and Mueller, 2010). *S. aequifasciatus* showed decreased CS and no change in COX capacity under elevated temperatures, suggesting the lack of temperature compensation in glycolytic capacity; these results confirm the importance of LDH and anaerobic metabolism in addressing the energetic imbalances induced by elevated temperatures. Specimens of *S. aequifasciatus*, however, showed increases in both CS and COX activities after acclimation in the presence of MPs, indicating a greater aerobic metabolism capacity for energy production. Thus, changes in metabolic pathways in response to elevated temperatures and MPs were evident during the early life stages of *S. aequifasciatus*; juveniles exposed to elevated temperatures and MPs relied on anaerobic glycolysis for energy production, and they also depended on aerobic metabolism in the presence of MPs.

5. Conclusion

Neither survival rate nor body length of *S. aequifasciatus* juveniles were affected by MPs, elevated temperatures or their combination. Elevated temperatures resulted in an increase in the concentration of MPs in fish bodies. Exposure to MPs decreased the PEPP at ambient temperatures but not at elevated temperatures. Elevated temperatures, however, showed no effect on the PEPP but antagonistically acted with MPs, leading to similar predatory performance under present and future conditions. AChE activity was only affected by MPs and decreased in the presence of MPs. Trypsin activity was only influenced by MPs and decreased under the exposure to MPs. Both elevated temperatures and MPs alone increased the amylase activity but interacted antagonistically. ALP activity was affected by MPs or elevated temperatures alone and decreased when experiencing both stressors. Both elevated temperatures and MPs alone increased LDH activity; however, the interaction between the two stressors, cancelled activity but was still higher than in the present conditions. CS activity decreased with elevated temperature but increased with exposure to MPs. COX activity was only influenced by MPs and increased in the presence of MPs. Overall, these findings suggested that MPs showed a greater impact than elevated temperatures on the predatory performance, digestion and energy production of *S. aequifasciatus*. Nevertheless, juvenile survival and growth were minimally impacted, and thus, *S. aequifasciatus* is prepared to cope with the IPCC projections of elevated temperatures and MPs in the water.

Acknowledgements

The study presented in the manuscript was funded by the Shanghai Collaborative Innovation Center for Aquatic Animal Genetics and Breeding (ZF1206), China; the China Postdoctoral Science Foundation (2017M621433); and the Doctoral Scientific Research Foundation of Shanghai Ocean University (A2-0203-17-100306).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2017.12.010.

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